Comparative Transcriptional Profiling of Two Contrasting Rice Genotypes under Salinity Stress during the Vegetative Growth Stage[1][w]

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Rice (*Oryza sativa*), a salt-sensitive species, has considerable genetic variation for salt tolerance within the cultivated gene pool. Two *indica* rice genotypes, FL478, a recombinant inbred line derived from a population developed for salinity tolerance studies, and IR29, the sensitive parent of the population, were selected for this study. We used the Affymetrix rice genome array containing 55,515 probe sets to explore the transcriptome of the salt-tolerant and salt-sensitive genotypes under control and salinity-stressed conditions during vegetative growth. Response of the sensitive genotype IR29 is characterized by induction of a relatively large number of probe sets compared to tolerant FL478. Salinity stress induced a number of genes involved in the flavonoid biosynthesis pathway in IR29 but not in FL478. Cell wall-related genes were responsive in both genotypes, suggesting cell wall restructuring is a general adaptive mechanism during salinity stress, although the two genotypes also had some differences. Additionally, the expression of genes mapping to the *Saltol* region of chromosome 1 were examined in both genotypes. Single-feature polymorphism analysis of expression data revealed that IR29 was the source of the *Saltol* region in FL478, contrary to expectation. This study provides a genome-wide transcriptional analysis of two well-characterized, genetically related rice genotypes differing in salinity tolerance during a gradually imposed salinity stress under greenhouse conditions.

Salinity is a major problem for both irrigated and rainfed agriculture. Irrigated agricultural systems supply roughly one-third of the world's food supply (Munns, 2002). Therefore, there is a great urgency in addressing the problem of salinity, especially with an increasing global population. Salt stress also is a major problem for rainfed agriculture in coastal areas because of seawater ingress during high tide and the rising shallow saline groundwater, particularly during the dry season. The problem of salinity has been approached through better management practices and introduction of salt-tolerant varieties in the affected areas. Unfortunately, the use of improved irrigation management practices in salt-affected areas has generally proven to be uneconomical and difficult to implement on a large scale. Thus, genetic improvement of salt tolerance of major cereal crops like rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*), and barley (*Hordeum vulgare*) appears to be the most feasible and promising strategy for maintaining stable global food production.

Rice, the most important cereal crop in many parts of the world, is considered to be salt sensitive (Maas and Hoffman, 1977). Sensitivity of rice to salinity stress varies with the growth stage. In general, rice plants are very sensitive to salinity stress at young seedling stages and less so at reproduction (Flowers and Yeo, 1981; Lutts et al., 1995). From an agronomic point of view, tiller number and number of spikelets per panicle have been reported to be the most salinity-sensitive yield components in one genotype (Zeng and Shannon, 2000). These components are determined at vegetative and panicle initiation stages, respectively (Hoshikawa, 1989). In contrast, rice is considered to be relatively more salt tolerant at germination (Heenan et al., 1988; Khan et al., 1997). Seed germination is not significantly affected up to 16.3 dS m^{-1} (Heenan et al., 1988). Hence, improvement of salt tolerance of rice should target the specific growth stages that are more sensitive to salinity stress and can substantially affect grain yield.

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Some traditional cultivars and landraces of rice are more tolerant than many elite cultivars to various abiotic stresses. These resistant genotypes are considered to be good sources of tolerance traits. However, they generally have poor agronomic traits, such as tall plant stature, photosensitivity, poor grain quality, and low yield. One example of a traditional genotype that is tolerant to high salinity is the Indian landrace, Pokkali. Pokkali has been frequently used as a donor of salt-tolerance traits in breeding programs. The salt tolerance of Pokkali is usually attributed to both its capacity to maintain a low Na^+-to-K^+ ratio in shoot tissue and its faster growth rate under saline conditions. A recombinant inbred population was developed at the International Rice Research Institute (IRRI) using Pokkali and IR29, an improved *indica* cultivar currently used as a salt-sensitive standard (Bonilla et al., 2002). Several salt-tolerant and salt-sensitive recombinant inbred lines (RILs) were identified after screening for salinity tolerance (Gregorio et al., 1997). One of the lines identified from the RIL population, FL478, a F2-derived F8, has salt tolerance higher than or comparable to the tolerant parent, Pokkali. Line FL478 is reported to have good tillering capacity under salt stress (A. Ismail, unpublished data). Reduced tillering is one of the main phenotypes observed under salinity stress during the vegetative stage that affects final yield (Hoshikawa, 1989). The RIL FL478 also maintains a lower Na^+-to-K^+ ratio than both the parent lines (Gregorio et al., 2002; Suriya-arunroj et al., 2004; Table I). Based on its ability to tiller well and maintain high potassium content under salinity stress, FL478 appears to be a good candidate for salinity stress tolerance in rice, particularly at the vegetative stage of growth.

Even though rice is considered to be generally salt sensitive, there is genetic variation for salt tolerance at critical stages in the cultivated gene pool (Yeo and Flowers, 1982; Moradi et al., 2003). Utilization of this genetic variation to improve salt tolerance of rice may benefit from an improved understanding of response of rice to salinity stress. One aspect of response to stress is at the transcriptional level, which involves alteration in expression of genes (Tester and Davenport, 2003). Due to the large number of genes involved in the response to various abiotic stresses, microarrays are increasingly being used to monitor global gene expression changes in Arabidopsis (*Arabidopsis thaliana*; Seki et al., 2002), rice (Kawasaki et al., 2001), and other systems (Ozturk et al., 2002). The salinity response detected through microarrays is likely to have the following components: (1) adaptive response; (2) nonadaptive response; (3) response to salt injury; and (4) heritable responses conferring tolerance. These components can confound the development of a mechanistic understanding of heritable variation, which is fundamental to breeding for salt tolerance. Therefore, it is critical to use genotypes identified through careful salt-tolerance phenotyping. FL478 and IR29 are two phenotypically well-characterized, but closely related, rice genotypes with marked difference in salinity tolerance and are useful in this regard.

The recent completion of the rice genome sequence (2004), coupled with enhanced annotations of the rice genome (The Institute for Genomic Research [TIGR] rice pseudomolecules, release 3; www.tigr.org/tdb/e2k1/osa1) and a whole-genome microarray from Affymetrix, have provided us with an opportunity to study rice functional genomics using global expression profiling. Here, we present the results from a comparison

Table I. *Phenotypic responses of salt-tolerant FL478 and salt-sensitive IR29*

Both genotypes were characterized for their response to salinity stress by measurement of shoot Na^+ and K^+ concentrations, chlorophyll content, total anthocyanin content, net photosynthetic rate, stomatal conductance, and transpiration rate (T_r).

Shoot Ions[a]	Treatment	Shoot Na^+	Shoot K^+	Shoot Na^+-to-K^+ Ratio
		mmol kg^{-1}	*mmol kg^{-1}*	
	FL478 control	38.6 ± 1.0	1035.0 ± 43.3	0.04
	FL478 salt	253.6 ± 10.4	827.0 ± 31.0	0.31
	IR29 control	39.1 ± 1.8	1028.6 ± 61.7	0.04
	IR29 salt	336.0 ± 3.6	747.6 ± 27.2	0.44
Pigments[a]	Treatment	Chlorophyll *a*	Chlorophyll *b*	Anthocyanins
		mg d^{-1}m^{-2}	*mg d^{-1} m^{-2}*	*mg d^{-1} m^{-2}*
	FL478 control	2.85 ± 0.14	0.78 ± 0.15	0.28 ± 0.08
	FL478 salt	3.16 ± 0.34	0.77 ± 0.11	0.26 ± 0.04
	IR29 control	2.62 ± 0.06	0.63 ± 0.05	0.20 ± 0.02
	IR29 salt	3.10 ± 0.29	0.71 ± 0.11	0.24 ± 0.02
Photosynthetic[b]	Treatment	Photosynthesis	Conductance	T_r
		μmol m^{-2} s^{-1}	*mol water m^{-2} s^{-1}*	*mmol water m^{-2} s^{-1}*
	FL478 control	26.03 ± 3.99	0.95 ± 0.17	13.03 ± 2.05
	FL478 salt	25.88 ± 2.14	0.62 ± 0.10	9.33 ± 1.29
	IR29 control	24.48 ± 3.39	0.61 ± 0.16	9.39 ± 1.97
	IR29 salt	25.41 ± 3.68	0.47 ± 0.07	7.46 ± 1.18

[a]Results are mean values of three replicates ±SE. Each replicate was derived from two plants. [b]Results are mean values of six replicates ±SE.

of two *indica* rice genotypes, salt-tolerant RIL FL478 and its salt-sensitive parent IR29, under salinity stress during the vegetative stage of growth. These two genotypes have similar phenology and growth habits.

RESULTS

Phenotypic Variation between FL478 and IR29 under Salinity Stress

Genotypes FL478 and IR29 were evaluated for shoot Na^+ and K^+ at the same growth stage as that used for expression analysis (Table I). The shoot Na^+ concentration in FL478 was markedly lower than in IR29. Both genotypes exhibited a decrease in K^+ concentration under salinity stress. However, FL478 maintained higher levels of shoot K^+ compared to IR29. The K^+-to-Na^+ ratio was also more favorable in the tolerant FL478 relative to IR29. The chlorophyll and total anthocyanin concentrations were also determined for both genotypes under control and stressed conditions (Table I). Chlorophyll *a* and chlorophyll *b* levels increased under salt stress in both genotypes. However, total anthocyanin levels increased slightly in IR29 but decreased in FL478 under salt stress. Gas exchange measurements indicated that the net photosynthetic rate per unit area was comparable in both genotypes and did not show a change in response to salinity stress (Table I). However, stomatal conductance and transpiration rates were found to decrease in response to salinity in both genotypes. Salt-tolerant FL478 maintained significantly higher stomatal conductance and transpiration rates under control conditions and salinity stress when compared to IR29. Visual damage of salinity stress appeared on some leaves of IR29 approximately 34 to 36 d after planting. This damage was in the form of necrosis at about one-third of the length of a leaf from the tip. Similar damage was observed in FL478 40 to 42 d after planting and in relatively fewer leaves (data not shown).

Response of FL478 and IR29 at Expression Level to Salinity Stress

Genotypes FL478 and IR29 were cultured in sand tanks for 22 d after germination. A gradual salinity treatment was applied at the vegetative stage (Fig. 1; see ''Materials and Methods''). Plants were harvested 30 d after germination for expression analysis. Due to gradual imposition and a moderate level of salinity stress induced in the experiment, no visual differences were observed between control and stressed plants for tolerant and sensitive genotypes. To identify statistically significant differentially expressed genes, we used a combined criterion of 2-fold or more change and *t* test *P* value < 0.05. We used the same criteria for both FL478 and IR29 to obtain genes that have a significant response to salt-stress treatment. A total of 164 probe sets were up-regulated in FL478 under salinity stress. Some of the genes induced in FL478 are listed in Table II. A nearly equal number of probe sets were found to be significantly down-regulated under salinity stress in FL478. Using the same statistical criteria, a total of 456 probe sets were induced by salinity stress in salt-sensitive IR29, among which Table III lists certain broad categories and selected genes that fit into these categories. Complete lists of genes responsive to salinity stress in FL478 and IR29 are provided (see supplemental data). The number of probe sets whose expression was suppressed in IR29 under stress was 184. Surprisingly, only eight probe sets were induced in common between FL478 and IR29 during salinity stress. A comparison between lists of probe sets down-regulated by salinity stress in FL478 and IR29 yielded only two probe sets in common. Figure 2 shows the number of probe sets responding to stress (Fig. 2A) and the overlap between genotypes (Fig. 2, B and C). It is important to point out that there is not always a one-to-one correspondence between one probe set and one gene. The number of probe sets may reflect an overestimate of the actual number of genes induced. To check whether the number of salt stress-responsive probe sets obtained by our method was influenced by the stringency of our criteria, we performed a statistical analysis using a 1.5-fold lower cutoff change while keeping the same *P*-value threshold of 0.05. With the less stringent method, we obtained 259 probe sets induced in FL478, 682 in IR29, and 15 probe sets commonly induced. This indicated that the small overlap is independent of threshold stringency for filtering significantly changing probe sets. The remainder of this discussion is based on a 2-fold or more change combined with *t* statistics in this report.

The method for obtaining annotations for the salt-regulated probe sets is described in ''Materials and Methods.'' We found a significant percentage of probe sets annotated as hypothetical and unknown proteins from the rice genome. The gene lists were distinctly enriched for certain categories, such as flavonoid pathway and cell wall-related genes, in addition to typical abiotic stress-responsive genes. Thus, we decided to focus our comments on these specific pathways and categories to further illustrate the different responses between FL478 and IR29 to salinity stress.

Flavonoid Pathway Is Induced in Salt-Sensitive IR29

Flavonoids are a diverse group of secondary metabolites with a wide array of biological functions, including roles in stress protection (Winkel-Shirley, 2002). Flavonoids are synthesized via the phenylpropanoid pathway. Several genes encoding important enzymes involved in the flavonoid biosynthetic pathway were induced in the salt-sensitive genotype IR29 under salinity stress (listed in Table III). The flavonoid biosynthetic pathway is shown in Figure 3A. Genes that are significantly induced in IR29 in response to salinity and involved in biosynthesis are shown in boldface. These genes include Phe ammonia lyase 1 (PAL1), which catalyzes the conversion of Phe to cinnamate,

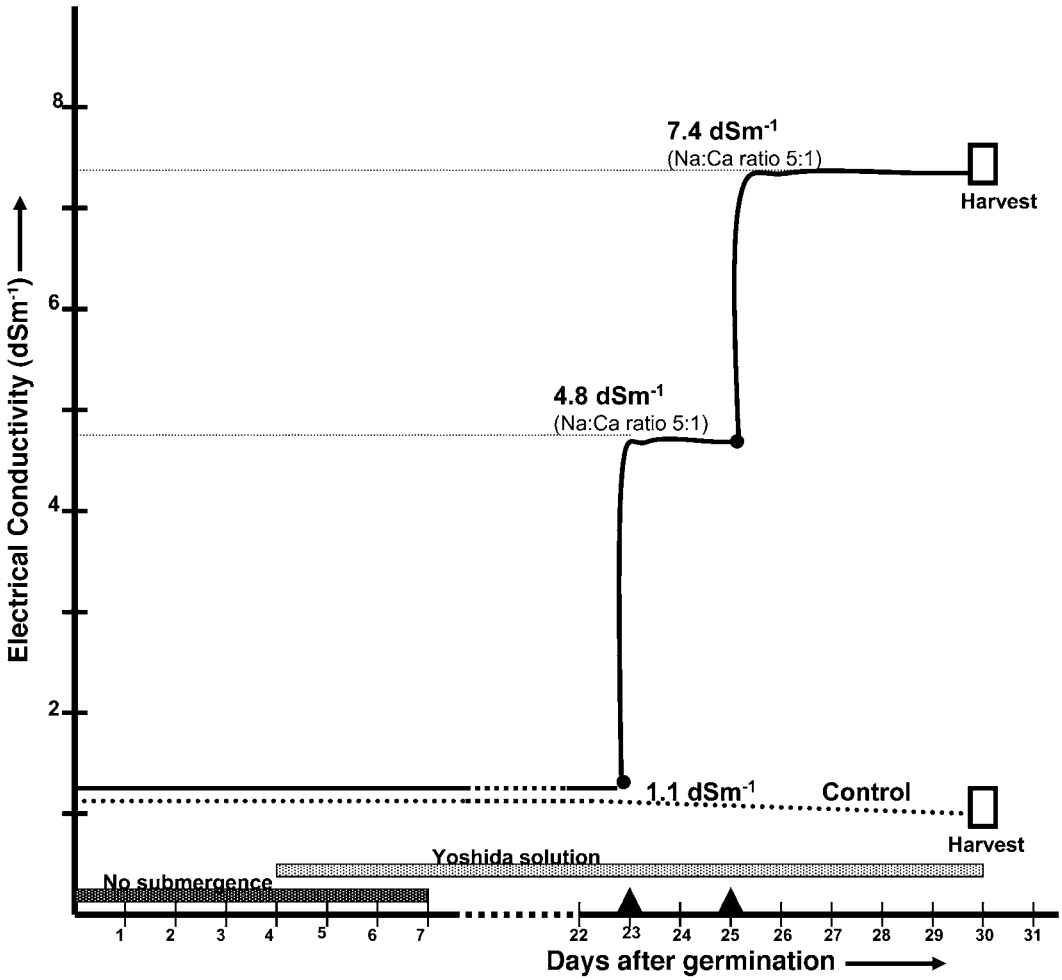

Figure 1. Experimental design of salinity stress treatment. The seedlings were submerged 7 d after germination. Yoshida solution was applied from day 4. A final salinity level of 7.4 dS m^{-1} was reached by a two-step addition of NaCl and $CaCl_2$ on days 23 and 25 after germination. A Na:Ca molar ratio of 5:1 was maintained during the addition of salts. Conductivity in the control tank is represented by a dotted line and in the treated tank by a solid line. Black circles represent addition of salt, and rectangles represent the harvest time points. The crown and growing point of the main shoot were harvested for expression analysis 30 d after germination.

and chalcone synthase (CHS), which is required for condensation of 4-coumaroyl-CoA and malonyl-CoA to yield naringenin chalcone. Chalcone isomerase (CHI), flavonoid 3′-hydroxylase (F3′H), and dihydroflavonol 4-reductase (DFR) were also up-regulated in response to salinity. Hierarchical clustering of probe sets induced in IR29 and representing genes involved directly or indirectly with the flavonoid pathway is displayed in Figure 3B. We found additional genes like *S*-adenosymethylthiopropylamine:carboxyl methyltransferase, flavin-containing monooxygenase, and glutathione *S*-transferase (GST), among others, to have a shared expression pattern in the heat map display. Figure 3C graphs the averaged expression of all the listed probe sets on a *y*-axis scale ranging from +3 to −3, and shows the increased level of expression in IR29 salt-stressed samples. None of the genes involved in the flavonoid pathway were induced at a statistically significant level in stressed samples of FL478. Therefore, induction of the flavonoid pathway appears to be a characteristic response of IR29 to salinity stress.

Salinity Induces Cell Wall-Related Genes in Rice

Plant cell walls play several critical roles during the life cycle of a plant, including response to environmental stresses. Primary cell walls are classified into two main groups, type I and type II, based on chemical structures of components, wall architecture, and biosynthetic processes (Carpita, 1996). Monocotyledonous cereals like rice have type II walls that are characterized by less pectin and higher amounts of phenylpropanoids in contrast to the type I cell wall present in Arabidopsis (Yokoyama and Nishitani, 2004). We found several cell wall-related genes responding to salinity stress in FL478 and IR29, which are listed in Table IV. Several contrasting trends were identified in the response of the two genotypes to stress in the

Table II. *Microarray data for selected genes induced by salinity stress in salt-tolerant genotype FL478*

Organism is the source of BLAST match for putative function annotation with *E* value shown. *P* value is the statistical significance of the differential expression for the probe set. Only selected probe sets with putative functions are listed. A complete list of genes responsive to salt stress in FL478 is provided (see supplemental data).

Putative Function	Probe Set ID	Organism	*E* Value	*P* Value
Transport				
Cyclic nucleotide-regulated ion channel	OsAffx.23819.2.S1_x_at	Rice	1e-60	0.034
Ankyrin repeat family protein	Os.49213.2.S1_at	Rice	1e-44	0.038
Glutamate receptor family protein	OsAffx.30030.2.S1_at	Rice	1e-23	0.003
Sodium transporter (HKT1)	Os.30563.1.S1_at	Rice	3e-19	0.001
ABC transporter family protein	OsAffx.2938.1.S1_at	Rice	8e-18	0.048
MATE efflux family protein	OsAffx.15672.1.S1_at	Arabidopsis	6e-70	0.031
Abiotic stress				
Protein phosphatase 2C	Os.18261.1.S1_at	Rice	2e-62	0.045
Protein peroxidase prx12 precursor	Os.47625.1.A1_s_at	Rice	4e-07	0.010
D-Man-binding lectin	Os.26941.1.A1_at	Rice	3e-44	0.000
WSI76 protein	Os.2677.1.S1_at	Rice	3e-42	0.008
Diacylglycerol kinase accessory	OsAffx.29212.1.S1_at	Rice	4e-59	0.006
Dehydration-responsive protein (RD22)	Os.28427.1.S2_a_at	Arabidopsis	3e-12	0.002
Late-embryogenesis abundant protein	Os.55959.1.S1_at	Arabidopsis	2e-10	0.002
Universal stress protein (USP) family	Os.55070.1.S1_at	Arabidopsis	6e-06	0.016
Dehydrin xero2 (XERO2)	Os.12633.1.S1_s_at	Arabidopsis	4e-05	0.015
Other genes				
Avr9/Cf-9 rapidly elicited protein	OsAffx.30030.2.S1_at	Rice	1e-100	0.002
CC-NBS-LRR resistance protein mla13	OsAffx.31356.1.S1_at	Rice	4e-33	0.004
Ser/Thr kinase-like protein	OsAffx.28531.1.S1_at	Rice	1e-99	0.042
DNA cytosine methyltransferase Zmet3	OsAffx.24938.1.S1_at	Rice	2e-86	0.006
Cytochrome P450 monooxygenase	OsAffx.12860.1.S1_at	Rice	3e-70	0.024
Iron-sulfur cluster-binding protein	OsAffx.25764.2.S1_at	Rice	2e-68	0.012
Lipid transfer protein	Os.6274.1.S1_at	Rice	7e-51	0.012
MADS-box protein-like	OsAffx.7652.1.S1_at	Rice	3e-26	0.000
FAR1 domain protein	OsAffx.27871.1.S1_at	Rice	3e-23	0.001
β-1,3-glucanase	Os.51679.1.S1_at	Rice	7e-11	0.047
V-type ATPase 116-kD subunit	Os.56862.1.S1_at	*Solanum demissum*	5e-06	0.049
Calcineurin-like phosphoesterase	OsAffx.17017.1.S1_at	Arabidopsis	7e-13	0.001
F-box family protein	OsAffx.20772.1.S1_at	Arabidopsis	9e-12	0.022
AGP19	Os.57128.1.S1_at	Arabidopsis	1e-07	0.0005
LOB domain protein	Os.10351.5.S1_x_at	Arabidopsis	5e-006	0.014

context of genes involved in cell wall construction, modification, and maintenance of cell wall architecture. One of the genes induced in IR29 encodes a cellulose synthase-like protein (OsCslF1), which may have a role in catalyzing the biosynthesis of hemicellulose backbones (Richmond and Somerville, 2001). Also up-regulated were genes for structural proteins, such as hydroxyl Pro-rich glycoprotein, Pro-rich protein, and Gly-rich proteins. However, hydroxyl Pro-rich glycoprotein and Pro-rich protein were down-regulated in FL478 under salinity stress. Expansins are another set of genes involved in cell wall expansion (Cosgrove et al., 2002). Since salinity is known to cause reduction in cell growth, it was not surprising to find the expression of β-expansin (EXPB2) suppressed in both FL478 and IR29 under stress conditions. Data from real-time quantitative PCR for the expression levels of EXPB in both genotypes agree with array data (Supplemental Table I). Another gene, arabinogalactan-protein (AGP9) is commonly down-regulated in both genotypes. A related gene encoding AGP19 was up-regulated in FL478. A gene for invertase/pectin methylesterase inhibitor family protein, which inhibits pectin metabolism, was induced in FL478 but down-regulated in IR29. Based on our data, it appears that salinity stress results in major cell wall restructuring in both salt-sensitive and salt-tolerant genotypes, including some commonalities and some differences in cell wall adaptations.

Physical Distribution of Loci Up-Regulated during Salinity Stress

We investigated the chromosome distribution of probe sets induced in IR29 and FL478 under salinity stress. Some of the stress-induced genes are clustered in very close proximity. A formula was developed to associate a probability value to these clusters (see "Materials and Methods"). These probability values are listed in Supplemental Table II. We found significant clusters on chromosomes 1, 6, and 7 for IR29 stress-induced genes. Of special note are a six-gene cluster on chromosome 1 (13.55–14.72 Mb), a five-gene

Table III. *Microarray data for selected genes induced by salinity stress in salt-sensitive genotype IR29*

Headings as in Table II. A complete list of genes induced in IR29 is provided (see supplemental data).

Putative Function	Probe Set ID	Organism	*E* Value	*P* Value
Transport				
ABC transporter family protein	OsAffx.29710.1.S1_x_at	Arabidopsis	8e-38	0.005
Sodium transporter (HKT1)	Os.57530.1.S1_at	Arabidopsis	1e-30	0.034
High-affinity potassium transporter 2	Os.30563.1.S1_at	Rice	3e-36	0.035
Mannitol transporter	Os.53546.1.S1_s_at	Arabidopsis	7e-14	0.019
Major facilitator superfamily antiporter	Os.21635.1.S1_at	Rice	6e-17	0.033
Transcription factors				
MADS-box protein	Os.320.2.S1_a_at	Rice	4e-70	0.028
Dehydration-induced myb protein Cpm7	OsAffx.14536.1.S1_at	Rice	4e-22	0.023
AP2 domain-containing trans. factor	OsAffx.15338.1.S1_at	Arabidopsis	1e-15	0.043
Helix-loop-helix DNA-binding domain	Os.27705.1.S1_a_at	Rice	3e-20	0.013
Zinc finger protein	Os.51002.1.S1_at	Rice	3e-90	0.021
Myb-like DNA-binding domain	Os.2867.1.A1_at	Rice	1e-17	0.028
Abiotic stress				
Mannose-binding rice lectin	Os.46023.1.S1_at	Rice	3e-64	0.031
Heat shock protein 16.9 kD	Os.14405.1.S1_at	Rice	1e-35	0.017
Dehydrin (RAB18)	Os.3419.1.S1_a_at	Arabidopsis	4e-08	0.030
α-Galactosidase	Os.17491.1.S1_at	Rice	3e-56	0.002
Group 3 LEA protein	Os.12551.1.S1_s_at	Rice	1e-18	0.024
Heat shock factor	Os.27176.1.S1_at	Rice	3e-10	0.007
Defense proteins				
Receptor kinase-like protein, Xa21	Os.26887.1.A1_at	Rice	4e-11	0.040
RPM1 like protein	OsAffx.29090.1.S1_at	Rice	6e-98	0.040
Bowman Birk trypsin inhibitor	Os.37729.1.S1_s_at	Rice	3e-30	0.026
β-Galactosidase	Os.56856.1.S1_at		1e-28	0.042
Pathogen-responsive α-dioxygenase	Os.2371.1.S1_at	Arabidopsis	3e-48	0.020
Wound-responsive family protein	Os.6764.2.S1_at	Arabidopsis	5e-22	0.034
Flowering				
Floral homeotic protein AGAMOUS (AG)	Os.320.2.S1_a_at	Arabidopsis	1e-22	0.028
Flowering locus T protein (FT)	OsAffx.15765.1.S1_at	Arabidopsis	7e-42	0.031
Anthocyanin biosynthesis				
Flavin-containing monooxygenase	Os.5265.1.S1_x_at	Rice	6e-42	0.033
Flavanone 3-hydroxylase	Os.10510.1.S1_at	Rice	5e-49	0.040
Flavonol 3-sulfotransferase	Os.46538.1.S1_at	Rice	3e-76	0.002
CHI	Os.9929.1.S1_at	Rice	7e-79	0.012
Flavonol 3-sulfotransferase	OsAffx.29191.1.S1_at	Rice	2e-84	0.017
PAL1	Os.27728.1.S1_at	Arabidopsis	8e-33	0.000
Flavonol 3-*O*-methyltransferase	OsAffx.13783.1.S1_at	Arabidopsis	2e-14	0.020
CHS	Os.12416.1.S1_at	Arabidopsis	6e-20	0.022
DHR	Os.23101.1.A1_at	Arabidopsis	6e-19	0.024
F3′H	Os.8511.1.S1_s_at	Arabidopsis	2e-27	0.030

cluster on chromosome 1 (42.11–42.44 Mb), a six-gene cluster on chromosome 6 (12.19–12.57 Mb), and a dense four-gene cluster on chromosome 7 (24.45–24.48 Mb). We performed expression-based hierarchical clustering of the genes in these physical clusters (see "Materials and Methods"). Interestingly, three of the four genes belonging to the physical cluster on chromosome 7 (22.6 kb) are in the same expression branch. A similar comparison of FL478 stress-induced genes yielded physical clusters of three genes each on chromosomes 5, 8, 10, and 11. Details of these physical clusters from both genotypes are listed in Supplemental Table III. We did not find any significant physical clusters among stress-down-regulated genes in either of the genotypes. These results suggest that several regions of the rice genome contain coordinately regulated genes associated with a response to salinity.

Gene Expression in the *Saltol* Region

The most prominent quantitative trait locus (QTL) for salt tolerance was previously mapped to rice chromosome 1 using an F2-derived F8 RIL population obtained from Pokkali × IR29. FL478 is one of these F8 RILs. Pokkali, the salt-tolerant parent, was the source of positive alleles for this major QTL (Bonilla et al., 2002), which accounted for high K^+ absorption, low Na^+ absorption, and low Na^+-to-K^+ ratio under

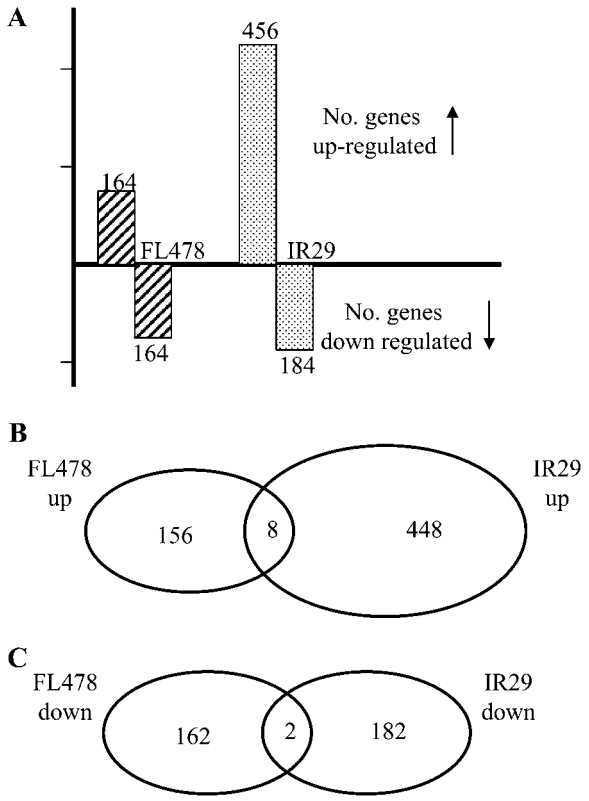

Figure 2. Number of differentially expressed probe sets. A, The histogram shows the total number of probe sets up- or down-regulated in FL478 and IR29 in response to salinity stress at a level of 2-fold or more and a P value < 0.05. B, Venn diagram illustrates the probe sets induced in either or both genotypes. C, Venn diagram illustrates the number of probe sets down-regulated under salinity stress. It is important to point out that a single gene described on the basis of best BLAST hits against the TIGR rice database is in many cases represented by multiple probe sets. Therefore, the probe set overlap between FL478 and IR29 is an underestimation of the number of shared differentially expressed rice gene models.

salinity stress (Gregorio et al., 2002). This QTL, called *Saltol*, is flanked by markers RM23 and RM140 (Gregorio et al., 2002). Other investigators (Lin et al., 2004) have identified a major QTL for high shoot K^+ under salinity stress in the same region of chromosome 1. Considering the consistent emergence of this region in independent salt-tolerance-related QTL studies, we paid special attention to the probe sets representing this region. We obtained the chromosomal positions of markers flanking the *Saltol* region and the QTL identified by Lin et al. (2004) by searching the Gramene database (www.gramene.org). Markers associated with the *Saltol* region and K^+ concentration QTL are summarized in Figure 4. This region roughly extends from 9.8 to 12.2 Mb on the short arm of chromosome 1. The rice gene models in this region corresponding to probe sets that were stress induced in IR29 are displayed in Figure 4C. Selected probe sets localizing to this region are listed in Supplemental Table IV. Probe set Os.30563.1.S1_at, which maps to rice locus Os01g20160 (11.4 Mb), is a cation transporter induced by salt stress in both genotypes. The increase of Os01g20160 expression in FL478 from GeneChip data is 5.9-fold compared to a 3.8-fold change in IR29. The difference appears to be somewhat greater from real-time quantitative PCR (15 for FL478 versus 6.5 for IR29; Supplemental Table I). We also found a tight cluster of four genes induced in IR29 localizing around 14.4 Mb on chromosome 1, which seems to be slightly proximal to the *Saltol* locus. We determined the source of the *Saltol* region in FL478 using a method developed for detecting single-feature polymorphisms between genotypes using RNA expression data (Cui et al., 2005). Results from this analysis indicated that IR29 was the contributor of the *Saltol* region in FL478. This implies that the salt tolerance of FL478 is most likely derived from QTL for salt tolerance localizing to a non-*Saltol* region of Pokkali.

DISCUSSION

In this study, we applied genome-scale gene expression analysis to two well-characterized rice genotypes currently being used as standards in several salinity stress studies (Gregorio et al., 2002; Suriya-arunroj et al., 2004; Zeng, 2004). The *indica* rice genotypes FL478 and IR29 differ in salt tolerance even though they are likely to share at least 50% of their genome, as IR29 is one of the parents used to develop the RIL population to which FL478 belongs. The results from our study demonstrate that the response of FL478 and IR29 to salinity stress is strikingly different. The overriding feature of the response of IR29 to salinity stress is the induction of transcription of a relatively large number of genes. Since FL478 is salt tolerant and maintains a low Na^+-to-K^+ ratio in shoot tissue, it may be relatively less stressed at the cellular level compared to IR29 and thus have a more limited response of the transcriptome. Collectively, these data indicate that the two genotypes are quite different from a transcriptional perspective.

Flavonoids and Salinity Stress

Flavonoids are a functionally diverse group of secondary products with roles in pigmentation, plant-microbe interaction, and reproduction. Flavonoids have also been linked to defense against various stresses, such as pathogens, wounding, and UV light damage. In our experiment, the flavonoid pathway was induced during salinity stress in salt-sensitive IR29, but not in FL478. The exact role flavonoids are playing in the sensitive line during salinity stress is not certain. However, we note that a similar case of induction of flavonoid biosynthetic pathway genes was reported in an ozone-sensitive but not in a resistant bean genotype during ozone treatment (Paolacci et al., 2001). The authors also reported sustained induction of certain antioxidant enzymes like peroxidases and superoxide dismutase in the ozone-sensitive genotype. We also

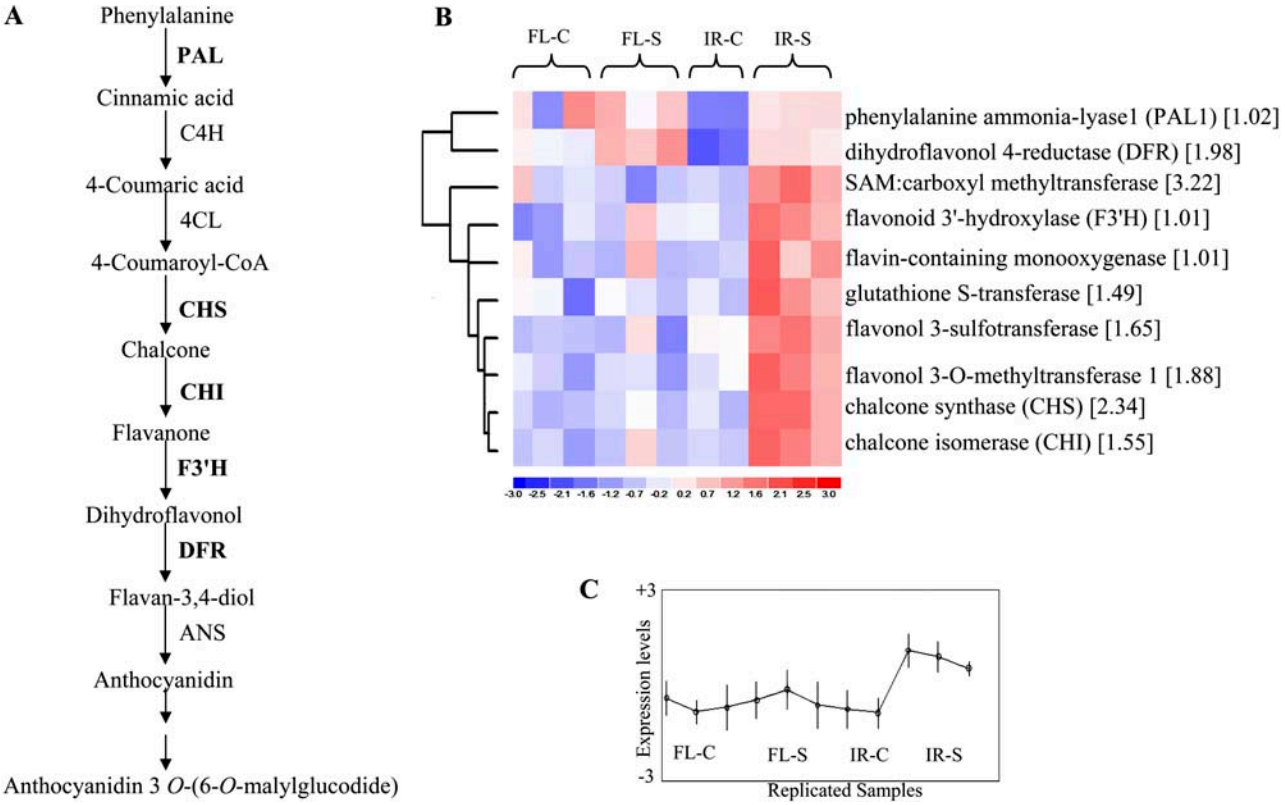

Figure 3. Flavonoid biosynthesis pathway induced in IR29 under salt stress. A, A simplified scheme for anthocyanin and flavonoid biosynthesis. Enzymes encoded by genes that are induced during salinity stress in sensitive genotype IR29 are in boldface. C4H, Cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; F3H, flavanone 3-hydroxylase; ANS, anthocyanidin synthase. B, The heat map display of genes related to the flavonoid pathway using hierarchical clustering. FL-C, FL478 control; FL-S, FL478 salinity stress; IR-C, IR29 control; IR-S, IR29 salinity stress. The difference in mean expression between control and stress conditions for IR29 ($\log_2$) is listed in square brackets. The expression values of the probe sets are scaled within a range from +3 (red) to −3 (blue). Red represents up-regulation, and blue represents down-regulation. The averaged expression value for each replicate from each genotype and treatment is shown in C. The vertical line through each of the plotted points represents error for the expression value. Expression values on the *y* axis are scaled from +3 to −3, corresponding to the heat map in B. The probe sets corresponding to the genes on the heat map are as follows: PAL1 (Os.27728.1.S1_at), DFR (Os.23101.1.A1_at), SAM:carboxyl methyltransferase (Os.11812.1.S1_at), flavonoid 3-hydroxylase (Os.10510.1.S1_at), flavin-containing monooxygenase (Os.5265.1.S1_x_at), GST (Os.13014.1.S1_a_at), flavonol 3-sulfotransferase (OsAffx.29191.1.S1_at), flavonol 3-*O*-methyltransferase (OsAffx.13783.1.S1_at), CHS (Os.12416.1.S1_at), and CHI (Os.9929.1.S1_at).

observed multiple peroxidases induced in IR29, but not in FL478. Flavonoids are also considered to play a role in UV protection in epidermal cells of leaves and in susceptible tissue like pollen and the apical meristem (Winkel-Shirley, 2002). Direct evidence supporting a role of flavonoids in UV protection was provided by a UV hypersensitive Arabidopsis mutant defective in CHS or CHI (Li et al., 1993). Another UV hypersensitive mutant of Arabidopsis, *uvs66*, was characterized to be hypersensitive to salinity stress also (Albinsky et al., 1999). The regulation of *RAB18* (responsive to abscisic acid, but not to NaCl, in wild type) was characterized in *uvs66*. *RAB18* was induced in *uvs66* by salinity stress. Interestingly, *RAB18* was also induced in IR29 during salinity stress. On a speculative note, it seems that the common thread connecting flavonoid pathway induction during salinity stress, ozone stress, and UV stress may be oxidative stress. This is based not only on our data reported here, but also on various other reports (Sandermann et al., 1998; Gould et al., 2002; Tester and Davenport, 2003).

Genes Regulated by Salinity Stress in Rice

Our data showed some well-characterized salt stress-responsive genes of rice (e.g. *salT*), which are induced in salt-sensitive IR29 but not in the more tolerant FL478. Rice locus Os01g25280 is annotated as a putative *salT* gene with a jacalin-like lectin domain. The *salT* gene was isolated and characterized from rice root tissue upon treatment with salt (Claes et al., 1990). It is also induced by abscisic acid and jasmonic acid besides salt stress (Moons et al., 1997). The transcript abundance of the putative *salT* gene is increased in IR29, but not in FL478, under salinity stress. Another group of genes typically responsive to salt stress are GSTs, which contribute to cellular survival under oxidative stress. The rice locus Os10g38140 encodes

Table IV. *Microarray data for cell wall-related genes responsive to salinity stress in genotypes FL478 and IR29*

Headings are as in Tables II and III. Multiple probe sets may have the same or similar annotations (e.g. Os.22927.1.S1_at is induced in IR29 and Os.51735.1.S1_s_at is repressed in FL478; both probe sets have similar annotations).

Putative Function	Probe Set ID	*E* Value	*P* Value
IR29 up-regulated			
Esterase/lipase/thioesterase-like protein	OsAffx.23399.1.S1_at	1e-85	0.048
Cellulose synthase-like protein (OsCslF1)	Os.52482.1.S1_at	9e-75	0.020
Glycine-rich protein	Os.49289.1.S1_x_at	1e-74	0.009
Xyloglucan endotransglycosylase	Os.27205.1.S1_at	2e-44	0.043
Hydroxycinnamoyl transferase	OsAffx.27513.1.S1_s_at	1e-43	0.034
Extensin-like cell wall protein	Os.56224.1.S1_at	5e-42	0.016
PAL	Os.27728.1.S1_at	4e-39	0.000
Vegetative cell wall protein	Os.49602.1.A1_at	5e-38	0.031
Pro-rich family protein	Os.56340.1.S1_at	3e-08	0.015
Polygalacturonase-inhibiting protein 1 (PGIP1)	OsAffx.26677.1.S1_x_at	4e-30	0.043
Hydroxyl Pro-rich glycoprotein family protein	Os.22927.1.S1_at	1e-22	0.007
Acidic endochitinase (CHIB1)	OsAffx.26914.1.S1_at	3e-23	0.013
AGP18	Os.56224.1.S1_at	3e-08	0.016
IR29 down-regulated			
AGP9	OsAffx.4155.1.S1_at	1e-05	0.037
Invertase/pectin methylesterase inhibitor family	OsAffx.8723.1.S1_at	5e-06	0.005
EXBP2	Os.2373.1.S1_at	5e-11	0.048
FL478 up-regulated			
AGP19	Os.57128.1.S1_at	1e-07	0.000
Invertase/pectin methylesterase inhibitor family	OsAffx.15088.1.S1_at	1e-07	0.006
β-1,3-glucanase	Os.12633.1.S1_s_at	7e-11	0.015
FL478 down-regulated			
Pectin esterase family protein	Os.33673.1.A1_at	2e-06	0.034
AGP9	OsAffx.9656.3.S1_x_at	1e-07	0.002
Hydroxyl Pro-rich glycoprotein family	Os.51735.1.S1_s_at	1e-05	0.031
Pro-rich family protein	OsAffx.27023.1.S1_x_at	1e-05	0.028
EXBP2	Os.2405.1.S1_at	2e-25	0.001

for a GST and is induced in IR29 but not in FL478. Rice locus Os07g48830 encodes for WSI76 protein, a galactinol synthase that is induced in FL478 under salinity stress. The transcript of galactinol synthase was reported to accumulate in rice seedlings in response to chilling (4°C) treatment and osmotic stress (Takahashi et al., 1994). At least two galactinol synthase genes are known to be induced in response to salt stress in Arabidopsis (Taji et al., 2002). Galactinol synthase is believed to catalyze the committed step in raffinose family oligonucleotide biosynthesis (Smith et al., 1991) and is involved in regulation of carbon partitioning between Suc and raffinose family oligonucleotides (Saravitz et al., 1987). Galactinol synthase is not induced significantly in IR29; however, other genes with a putative role in movement and sequestering of resources like vegetative storage protein, mannitol transporter, and a monosaccharide transporter are up-regulated in IR29 under stress.

Salt tolerance in monocots is generally associated with the ability of plants to exclude Na^+ from the shoot tissue (Tester and Davenport, 2003). In rice, the concentration of Na^+ and Na^+-to-K^+ ratio are correlated with the seedling growth and grain yield under saline conditions (Flowers and Yeo, 1981). Our results indicate that FL478 accumulates lower Na^+ and higher K^+ in shoot tissue and maintains a more favorable Na^+-to-K^+ ratio compared to IR29 during salt stress (Table I). The salt-tolerant landrace Pokkali is reported to accumulate lower levels of Na^+ relative to IR29 under salinity stress (Golldack et al., 2003; Zeng, 2004). In another comparative evaluation of salt tolerance of various rice genotypes, FL478 was found to have a lower Na^+-to-K^+ ratio than Pokkali (Suriya-arunroj et al., 2004). We identified multiple ion transporters responding to our stress treatment in FL478 and IR29. Notably, a sodium-potassium cotransporter (HKT1) was up-regulated in both FL478 and IR29. Expression of HKT1 in root tissue is well characterized (Gassmann et al., 1996; Laurie et al., 2002). Under high external Na^+, it becomes a low-affinity sodium transporter (Rubio et al., 1995). A gene encoding for a protein with ankyrin repeats was up-regulated in FL478. The ankyrin repeat is a common protein-protein interaction motif associated with diverse functional roles, including ion transport. The ankyrin repeat domain is present in some of the plant inward-rectifying potassium channels (Cao et al., 1995). These channels are involved in the low-affinity K^+ transport and are characterized by a cyclic nucleotide-binding domain (Fox and Guerinot, 1998). Interestingly, a cyclic nucleotide-regulated ion channel (At5g54250) represented by

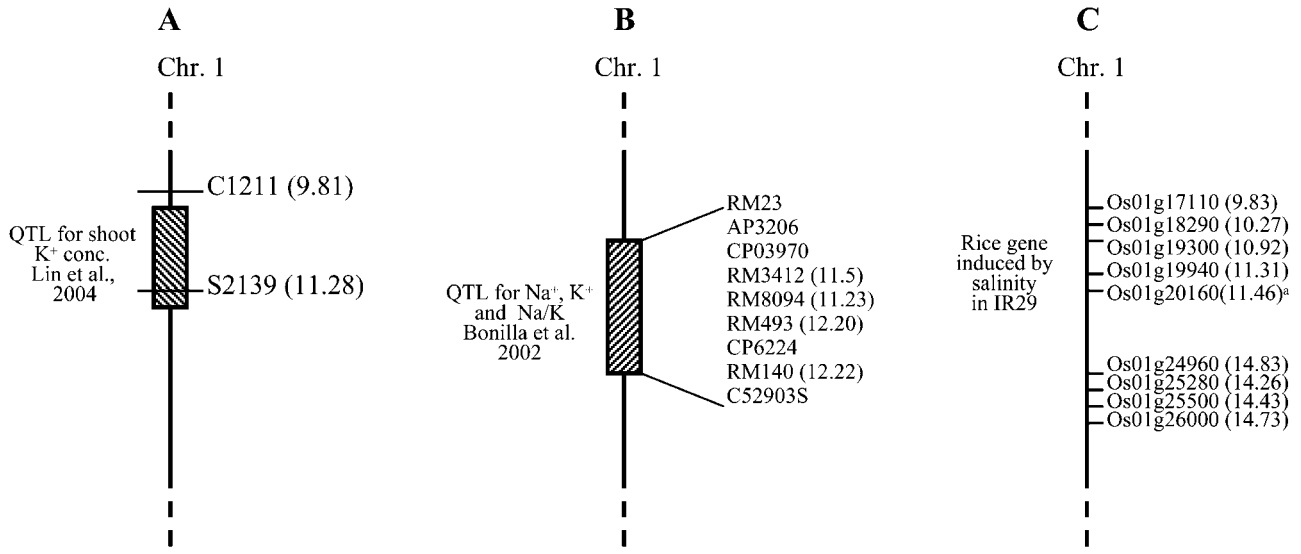

Figure 4. Chromosome 1 segment associated with a major QTL for salinity tolerance. A, Lin et al. (2004) described a QTL, qSKC-1, denoting shoot potassium concentration under salt stress. This QTL localizes between 9.81 and 11.28 Mb based on approximation of associated marker locations. B, An overlapping region flanked by markers RM23 and C52903S was identified by Bonilla et al. (2002) contributing to three quantitative traits (low Na^+ absorption, high K^+, and a low Na^+-to-K^+ ratio) in a mapping population derived from a cross between Pokkali and IR29. Additional markers were generated by E.B. Tumimbang, D.L. Adorada, J. Niones, F. Elahi, Z. Seraj, J. Dvorjak, and G.B. Gregorio. C, Rice genes induced in IR29 that colocalize to the same region under salt stress in our stress treatment. Rice gene Os1g20160 (11.46 Mb) is a cation transporter that is induced in both FL478 and IR29 under salinity stress.

OsAffx.23819.2.S1_x_at is also induced in FL478. A high-affinity potassium transporter, HAK5 (OsAffx. 3426.1.S1_at), is down-regulated in FL478. HAK5 is induced in shoot tissue of K^+-starved plants (Rubio et al., 2000). The ankyrin repeat characterized protein is potentially a differentiating factor that may contribute to the low Na^+-to-K^+ ratio of FL478 relative to IR29.

It has been suggested that the cell wall, plasma membrane, and cytoskeleton of a plant cell behave as an integrated entity (Baluska et al., 2003). Therefore, stress-induced changes in the state of the plasma membrane are believed to alter the cell wall and cytoskeleton (Kacperska, 2004). Our data are consistent with this hypothesis. Genes associated with cell wall regulation responded to our salinity stress treatment in rice. Genes encoding AGPs, cellulose synthases, and receptor-like protein kinases have been proposed to regulate the plasma membrane-cell wall interface (Kohorn, 2000). All of these proteins are bound to the plasma membrane and extracellular carbohydrate. More recently, arabinogalactans and formins that are responsive to salinity stress have been proposed as candidates for plant linkers between the cell wall and the cytoskeleton (Baluska et al., 2003). As yet, the implications of changes in transcript levels of different arabinogalactans up- or down-regulated in FL478 and IR29 during the stress treatment are unknown. However, their modulation points to perturbance in the levels of AGPs during salinity stress in both genotypes. Reduction in the level of AGPs in apoplasts is reported to inhibit cell expansion and is a general response of plant cells to salinity (Zhu et al., 1993; Majewska-Sawka and Nothnagel, 2000).

Chromosomal Distribution of Salinity-Responsive Genes

We found several instances where coregulated genes are tightly clustered in the rice genome. Coexpressed clusters were more prominent on IR29 than FL478, but this difference may simply be attributed to a greater number of responsive genes in the sensitive genotype. Recent studies provide several examples of neighboring genes in eukaryotic genomes having similar expression patterns (Spellman and Rubin, 2002). Using expression-based hierarchical clustering criteria, we found an instance on chromosome 7 where physically clustered genes grouped to the same branch. However, it was not a general trend among all the clusters identified. The question that emerges is whether there is an advantage for the genome to maintain these presumably functionally related gene clusters. One model proposes it to be due to incidental expression of genes close to each other not being discriminated by the regulatory machinery (Li et al., 2005). An alternative model suggests the possibility of clusters corresponding to regions of active chromatin (Chang et al., 2004). Indiscriminate induction of genes in the clusters is probably not the case since we observed that some down-regulated genes comingled with the induced genes within this cluster.

Differentially Expressed Genes and QTL Association

We were able to assign chromosomal positions to a total of 601 induced probe sets that fulfilled the threshold value for being differentially expressed in FL478 or IR29. To further investigate the possibility

that some of the genes represented by these probe sets play an important role in response of rice to salinity stress, we compared the genomic locations of these gene models with those of reported rice QTL for salinity tolerance (Bonilla et al., 2002; Lin et al., 2004). Bonilla et al. (2002) mapped a major salt-tolerance QTL, *Saltol*, on chromosome 1 in the region flanked by markers RM23 and RM140. Lin et al. (2004) also associated this region with shoot K^+ concentration. We focused on this region extending roughly from 9 to 12 Mb in order to identify possible candidate genes that contribute to the salt-tolerance QTL localized to this region by multiple studies. A total of 477 rice loci have been assigned to this region by TIGR rice assembly version 3, and 604 probe sets of the rice genome array localize to this region based on best BLAST hit results against the rice genomic sequence. Our microarray analysis provides expression data for most of the genes in this region. We found a higher number of differentially expressed genes from this region in IR29 (total of 10) than in FL478 (two genes). The transcript level of Os1g20160 (11.46 Mb), encoding a cation transporter, is increased in both FL478 and IR29. The up-regulation in FL478 is, however, to a higher level than IR29. We do not know whether the difference in fold change is due to allelic variation at this locus between the two genotypes. The possibility of identifying tolerance conferring genes of FL478 by sampling at one time point and tissue type is limited. However, genes that localize to a QTL region and have transcript abundance associated with the QTL trait become interesting candidates for further detailed analysis. Another approach using single feature polymorphism marker analysis revealed IR29 to be the source of the *Saltol* region in FL478. Since FL478 must carry a favorable combination of positive alleles for salt-tolerance genes from both parents, it was somewhat surprising to find the sensitive parent contributing this particular region, which has come to the fore as a major salt-tolerance QTL in several studies. However, the concept of some of the most favorable QTL alleles residing in a genotype that does not have the most favorable phenotype has many precedents (Tanksley, 1993).

MATERIALS AND METHODS

Plant Materials for Expression Studies

Seeds of rice (*Oryza sativa*) genotype IR29 and FL478 were obtained from G.B. Gregorio at IRRI in The Philippines. The rice genotype FL478 is also known as IR66946-3R-178-1-1. Seed increase and expression studies were conducted at George E. Brown, Jr., Salinity Laboratory, Riverside, CA.

Plant Culture and Salinity Treatment

The experiment was conducted in the greenhouse at Riverside, CA (33°58′24′′ N latitude, 117°19′12′′ W longitude) between July and August. Plants were cultured in tanks (122 × 61 × 46 cm) filled with sand and irrigated with nutrient solution (Yoshida et al., 1976). The nutrient solution consisted of NH_4NO_3 (1.43 mM), $NaH_2PO_4 \cdot 2H_2O$ (0.37 mM), K_2SO_4 (0.5 mM), $CaCl_2$ (1.00 mM), and $MgSO_4 \cdot 7H_2O$ (1.6 mM). Nutrient solution pH was maintained between 5.0 and 6.5 by adding sulfuric acid weekly. Irrigation solutions were prepared in 1,600-L reservoirs and pumped to provide irrigation to the sand tanks. Overflow irrigation was returned to the reservoirs through drainage by gravity. Each reservoir provided irrigation water to three sand tanks (replicates) three times daily for 30 min per irrigation cycle. Three rows of each genotype were planted per tank. An extra row at each end of the tank was used as the border row. The water level was maintained at the sand surface until emergence of the seedlings from the sand and then increased to 6 to 8 cm above the sand surface. Air temperature ranged from 32°C to 45°C during the day and from 19°C to 22°C during the night. Relative humidity ranged from 40% to 80%. Light averaged 1,104 μmol m^{-2} s^{-1} at noon. The seedlings were cultured in nutrient solution for 22 d after germination. Weak plants were removed from the tanks. Salinity treatment was applied by adding NaCl and $CaCl_2$ (5:1 molar concentration) in two steps over a period of 3 d to one reservoir (Fig. 1). Electrical conductivities (EC_w) of nutrient solutions were monitored using an EC meter daily (Hanna Instruments). The salinity in the treatment reservoir was allowed to stabilize for 5 d to a final electrical conductivity of 7.4 dS m^{-1}. The nonsaline control reservoir with Yoshida solution only had an EC_w of 1.1 dS m^{-1}.

Phenotypic Characterization and Gene Expression Studies

Plants were characterized for phenotypic responses to salinity stress on day 30. For phenotypic characterization of FL478 and IR29, shoot tissue was harvested for ion analysis. Plants were washed with deionized water, dried in a forced-air oven (70°C), and then ground into fine powder. Shoot Na^+ and K^+ concentrations were determined on nitric-perchloric acid digests by inductively coupled plasma optical emission spectrometry (ICP; Perkin-Elmer). Net photosynthetic rate per unit area and stomatal conductance of the youngest fully expanded leaf were measured between 10 AM to noon on day 30 after planting using a LI-COR 6400 photosynthesis system (LI-COR Biosciences). The following conditions for leaf gas measurements were used: photosynthetic photon flux density, 1,200 μmol m^{-2}; chamber CO_2 concentration, 380 μmol CO_2 mol^{-1}; leaf temperature, 27°C; and chamber vapor concentration, 20 mmol water mol^{-1}. Chlorophyll and anthocyanin levels of FL478 and IR29 were determined from control and stressed plants. Five leaf discs (diameter 5.1 mm) were taken from the youngest fully expanded leaf on the main shoot. The discs were placed in 5 mL of dimethyl sulfoxide for 24 h in the dark at room temperature (25°C) to extract leaf pigments (chlorophyll *a*, chlorophyll *b*, and anthocyanins). One milliliter of the pigment extract was then pipetted into a cuvette with a 1-cm light path and its absorbance was read at 470, 535, 648, and 664 nm using a Beckman DU7500 spectrophotometer (Beckman Coulter). Final pigment concentrations of chlorophyll *a* and chlorophyll *b* were calculated based on the absorbance and formula given by Chappelle et al. (1992). Final anthocyanin levels were obtained using the method of Fuleki and Francis (1968).

The plants were harvested on day 30 for RNA extraction. The main shoot was dissected to obtain the growing point and crown tissue, which was snap frozen. Approximately 12 plants were harvested per genotype per tank and tissue pooled to make one sample for RNA extraction. Two plants from each tank were allowed to grow to maturity to ensure that plants survive the imposed salinity stress.

RNA Extraction and Processing for GeneChip Analysis

RNA samples were processed as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical Manual; Affymetrix) at the DNA and Protein Microarray Facility at the University of California, Irvine, by Sriti Misra. Total RNA was initially isolated from frozen shoot tissue using TRIzol reagent. The RNA was purified using an RNeasy spin column (Qiagen) and an on-column DNase treatment. Eluted total RNAs were quantified with a portion of the recovered total RNA and adjusted to a final concentration of 1 μg/μL. All RNA samples were quality assessed prior to beginning target preparation/processing steps by running out a small amount of each sample (typically 25 to 250 ng/well) onto a RNA Lab-on-a-Chip (Caliper Technologies) that was evaluated on an Agilent bioanalyzer 2100 (Agilent Technologies). Single-stranded, then double-stranded cDNA was synthesized from the $poly(A)^+$ mRNA present in the isolated total RNA (10 μg total RNA starting material each sample reaction) using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) and poly (T)-nucleotide primers that contained

a sequence recognized by T7 RNA polymerase. A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction, using the Affymetrix GeneChip IVT labeling kit. Fifteen micrograms of the resulting biotin-tagged cRNA were fragmented to strands of 35 to 200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 10 μg of this fragmented target cRNA was hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 320) to probe sets present on an Affymetrix rice genome array. The GeneChip arrays were washed and then stained (streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450 followed by scanning on a GeneChip Scanner 3000.

Rice Genome Array

The rice genome array (Affymetrix) contains probe sets designed from approximately 48,564 *japonica* and 1,260 *indica* sequences. The sequence information for this array was derived from the National Center for Biotechnology Information (NCBI) UniGene Build number 52 (http://www.ncbi.nlm.nih.gov/UniGene), GenBank mRNAs, and 59,712 gene predictions from TIGR's osa1 version 2.0. Gene models that had any indication of transposable elements were removed from the list of TIGR predicted genes. The array is believed to represent about 46,000 distinct rice genes. About 26,000 of these are 3′-anchored Unigene expressed sequence tag and mRNA clusters, including known rice full-length cDNA clones, and 19,431 are solely from TIGR gene predictions. To obtain annotations for the salt-regulated probe sets, we extracted the target sequence of identified probe sets from the sequence information file (.sif) for the rice genome array. The target sequence extends from the 5′ end of the 5′-most probe to the 3′ end of the 3′-most probe. The target sequences were then searched using BLASTn against the TIGR rice pseudomolecules, release 3 (www.tigr.org/tdb/e2k1/osa1), and the TIGR Arabidopsis database, version 5 (Haas et al., 2005). These annotations for salinity stress-responsive probe sets are provided (see supplemental data).

Statistical Analysis of Array Data

The hybridization data were analyzed using GeneChip Operating Software (GCOS 1.2) and dChip (Li and Wong, 2001; http://www.dchip.org) software. The scanned images were examined for any visible defects. Satisfactory image files were analyzed to generate raw data files saved as CEL files using the default settings of GCOS 1.2 from Affymetrix. We used a global scaling factor of 500, a normalization value of 1, and default parameter settings for the rice genome array. The detection calls (present, absent, or marginal) for the probe sets were made by GCOS. Further analysis was done using dChip, which incorporates a statistical model for expression array data at the probe level. The dChip program was set to import GCOS signals. The normalization of all arrays was performed using an invariant set approach. We found the median intensity of all but one array comparable after the normalization procedure. For calculating the expression index of probe sets, we used the perfect match/mismatch model and opted for truncating the low-expression values to the tenth percentile of the probe set signals called absent. The expression values were $\log_2$ transformed after calculating the expression index. dChip was used for comparative analysis of samples from salt stress and control-treated plants for both genotypes. We identified the differentially expressed genes using the empirical criterion of more than 2-fold change ($E - B > 1$ or $B - E > 1$) and significant t tests of $P < 0.05$ based on three biological replicates. The baseline array, which is a control sample, is denoted by B, and salinity-treated experimental sample is denoted by E. The P values assigned to differentially expressed genes were used as a ranking criterion for ordering of up-regulated and down-regulated genes. We used the dChip feature to assess the false discovery rate in our dataset using a range of permutations (50–250). However, the false discovery rate estimates were not stable. This is likely due to the limited number of distinct permutations generated from our dataset.

We combined the lists of probe sets that were responsive to salt stress in FL478 and IR29 and used this list to perform unsupervised hierarchical clustering (Eisen et al., 1998) using dChip. The threshold for calling significant gene clusters was set to a P value of 0.001. Before clustering, the algorithm standardized (linearly scaled) the expression value of each of the listed probe sets in all samples to have a mean 0 and SD 1. These standardized values were then used to calculate correlations between probe sets and clusters, and serve as the basis for merging nodes. The distance between two neighboring probe sets was calculated using the Pearson correlation model and expressed as $1 - |r|$, which is the length of the stem connecting two branches where r denoted the Pearson correlation coefficient.

Real-Time, Quantitative PCR Validation

Expression levels of three genes, a cation transporter (Os01g20160), EXPB (Os10g40710), and CHS (Os11g32650), were analyzed using real-time, quantitative reverse transcription (RT)-PCR as a validation of microarray results. The *japonica* sequence of each gene was obtained from the TIGR rice database. These sequences were used to search for the corresponding *indica* sequences using the Beijing Genomics Institute-Rice Information System (BGI-RIS; http://rise.genomics.org.cn). Exonic sequences from each gene were used for the design of primers using Primer Express (Perkin-Elmer Applied Biosystems). The sequences for the forward and reverse primers are Os01g20160 (F-TTCATGGCGGTCAACTCGA, R-TTTGCTGGTGTTTGTCTTGGA), Os10g40710 (F-ATGAACTACTACCCCGTGGCC, R-TGGATGTCGATGATGCCG), and Os11g32650 (F-AGAAGGCGATCAAGGAGTGG, R-GCATCTTGGCGAGCTGGTAGT). The primer sequences for 18S rRNA (F-ATGATAACTCGACGGATCGC, R-CTTGGATGTGGTAGCCGTTT) were obtained from Kim et al. (2003) and used as an internal control. Total RNA from control and stressed samples of both genotypes was used for RT reactions using the TaqMan RT reagent kit (N808-0234; Applied Biosystems). A standard curve, composed of six points, was constructed from 2-fold dilutions of cDNAs. All real-time PCR reactions were performed using the ABI 7700 sequence detection system (Perkin-Elmer Applied Biosystems). Real-time amplifications were done using the SYBR Green PCR master mix (Applied Biosystems). The thermal cycling conditions were composed of 50°C for 2 min followed by an initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, then 60°C for 1 min. The experiments were performed in triplicate for each data point. Threshold cycles for each of the target genes and control were adjusted manually. Real-time PCR efficiencies (E) were calculated from the slopes of standard curves for each gene ($E = 10^{-1}/\text{slope}$). Samples from the unstressed group were selected as a calibrator. Gene expression in the salinity-treated samples was expressed relative to the calibrator and as a ratio to 18S rRNA using the formula (Pfaffl, 2001):

$$\text{Relative expression ratio} = (E_{\text{target}})^{\Delta \text{CP}_{\text{target(control - target)}}} / (E_{\text{18S}})^{\Delta \text{CP}_{\text{18S(control - target)}}},$$

where E_{target} is the real-time PCR amplification efficiency of the target gene transcript; E_{18S} is the real-time PCR amplification efficiency of 18S; and $\Delta \text{CP}_{\text{target(control - target)}}$ and $\Delta \text{CP}_{\text{18S(control - target)}}$ are the cycle threshold differences between the calibrator (the unstressed group) and the stressed group for the target gene and 18S rRNA, respectively. The Excel spreadsheet tool for this analysis was obtained from http://pathmicro.med.sc.edu/pcr/realtime-home.htm. The data from real-time quantitative PCR are provided in Supplemental Table I.

Physical Clusters of Salinity-Induced Rice Genes

To associate probability values with salinity-induced gene clusters, we developed a formula to account for the length (L) of chromosomes, total (t) number of gene models on individual chromosomes that are probed by the rice genome array, and the number of salt-induced genes (s) that are physically mapped to each chromosome. For this, each chromosome was divided into nonoverlapping windows of arbitrary length, 1 Mb. The maximum distance between neighboring genes to be included in a cluster was restricted to 500 kb. We assumed the colocalization of four or more genes within 1 Mb to be a significant cluster. We were interested in calculating the probability of an event where s genes induced by salinity map to a chromosome of length L Mb and have a total of t genes, such that four of the induced genes fall in a window of 1 Mb.

$$P = 4(t/L-4)/s(t-s)$$

The formula above assumes that gene density is uniform along the length of the chromosome. The probability that four genes from a 1-Mb window will be selected among s genes is $(4/s)$. Similarly, the probability that remaining $(t/L - 4)$ genes from the window will not be among the s genes selected is denoted by $(t/L - 4)/(t - s)$.

Data Availability

All microarray data from this work are available from NCBI GEO (www.ncbi.nlm.nih.gov/geo) under the series entry GSE3053.

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